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Rhodopsin and the others: a historical perspective on structural studies of G protein-coupled receptors

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Abstract

The role of rhodopsin as a structural prototype for the study of the whole superfamily of G protein-coupled receptors (GPCRs) is reviewed in an historical perspective. Discovered at the end of the nineteenth century, fully sequenced since the early 1980s, and with direct three-dimensional information available since the 1990s, rhodopsin has served as a platform to gather indirect information on the structure of the other superfamily members. Recent breakthroughs have elicited the solution of the structures of additional receptors, namely the β_1 - and β_2 -adrenergic receptors and the A_{2A} adenosine receptor, now providing an opportunity to gauge the accuracy of homology modeling and molecular docking techniques and to perfect the computational protocol. Notably, in coordination with the solution of the structure of the A_{2A} adenosine receptor, the first “critical assessment of GPCR structural modeling and docking” has been organized, the results of which highlighted that the construction of accurate models, although challenging, is certainly achievable. The docking of the ligands and the scoring of the poses clearly emerged as the most difficult components. A further goal in the field is certainly to derive the structure of receptors in their signaling state, possibly in complex with agonists. These advances, coupled with the introduction of more sophisticated modeling algorithms and the increase in computer power, raise the expectation for a substantial boost of the robustness and accuracy of computer-aided drug discovery techniques in the coming years.

Keywords

rhodopsin; adrenergic receptor; adenosine receptor; molecular modeling; docking; GPCR dimer; historical perspective

G protein-coupled receptors (GPCRs) are a large superfamily of proteins expressed on the plasma membrane that function as receivers for extracellular chemical or physical stimuli. Due to the vast physiological and pathophysiological implications of their signaling, GPCRs constitute particularly appealing targets for therapeutic interventions, with their modulators making up a large share of the current drug market.

For many years, there has been little knowledge on the structure of GPCRs. With crystallographic information available only for rhodopsin, homology modeling was widely applied to gather indirect information on the structure of other receptors. In this review, we provide a historical perspective on the role of rhodopsin as a prototypical GPCR, illustrating

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the massive amount of structural information about this protein that has been used as a platform for the study of the structure-function relationships of the whole superfamily. A schematic timeline is shown in Figure 1. As we will discuss, the structures of three additional members of the superfamily – namely the β_1 and β_2 -adrenergic receptors and the A_{2A} subtype of the adenosine receptors – have now been solved through crystallography, providing further insights into the three-dimensional topology of GPCRs (Figure 1). In light of these data, the generality of the structural information derived from rhodopsin and the substantial homology among the members of the superfamily has emerged more evident than ever, while some elements of variability have also been highlighted. The accelerated rate of the breakthroughs in GPCR structural studies promises the accumulation of a substantial amount of information in the years to come, with all the obvious consequent implications for pharmaceutical discovery and structure-based drug design.

Rhodopsin: the prototypical GPCR

Rhodopsin, a receptor activated by light photons and involved in visual signal transmission, has been the first receptor to be analyzed from a structural point of view and it still remains the best characterized. Although considered in many respects the prototypical GPCR, rhodopsin has a peculiarity that distinguishes it from the great majority of the superfamily members: the protein moiety, opsin, is covalently bound through a Schiff-base to the chromophore, a conjugated olefin known as retinal. The light driven isomerization of the latter is what triggers the activation of the receptor, which in turn, stimulates a guanine-nucleotide binding protein, or G protein, specialized in the transduction of the visual signaling – transducin (G_t) – leading to the activation of a phosphodiesterase that converts cyclic guanosine 3',5'-monophosphate (cGMP) to GMP.

In 1851, Heinrich Müller was the first to notice the red color of the retinal rod cells [1,2], but the seminal work on the effect of light on rhodopsin is now considered to be represented by papers published by Boll and Kühne in November 1876 and January 1877, respectively. Franz Boll, then a professor of physiology in Rome, was the first to observe that the color of rhodopsin bleaches in the light and regenerates in the dark and explicitly suggest a linkage between this phenomenon and the act of seeing [3-5]. Although in his initial communication to the Berlin Academy of Sciences, dating November 1876, he described the dark adapted rhodopsin as purple-red (*purpurrot*), in the full-length paper of 1877 Boll explained that it is, in fact, red and called it visual red (*Sehrot*). To his great disappointment, immediately after his 1876 communication, Wilhelm Kühne quickly entered the field of rhodopsin, to which he referred as visual purple (*Sehpurpur*), confirming some of Boll's findings and rectifying others. Chiefly, in opposition to Boll's initial hypothesis, Kühne observed that the purple color of the retina persists after death, unless it is exposed to light [6-9]. Boll became well aware of this fact soon after his communication, as he clarified in his 1877 paper, but his initial misinterpretation had already offered a pretext to Kühne to initiate his parallel studies, which led to numerous publications on the subject in the following years. Boll's work, instead, was interrupted by his premature death in Rome on December 19, 1879. Brief commentaries on Boll's and Kühne's discoveries have been translated into English by their contemporary Arthur Gamgee [10,11], while complete translations of Boll's 1877 and Kühne's 1879 papers have been published on the occasion of the 100th anniversary of their exceptional discoveries, by Ruth Hubbard and George Wald [5,9]. In the same commemorative issue of Vision Research, Baumann and Crescitelli provided insightful accounts of the research accomplishments and lives of Boll and Kühne [12-14], while another tribute to Kühne's life and accomplishments has been published in the Archives of Ophthalmology [15].

Following evidence that vitamin A, a retinoid alcohol, is important in vision, Wald demonstrated in 1935 that light promotes the dissociation of retinene, a carotenoid now known

as retinal, from the colloidal component of the visual pigment. Additionally, Wald demonstrated that vitamin A and retinene subsequently re-associate with the colloid component to reform visual purple, thus completing the cycle of the visual process. Moreover, Wald came to realize that “visual purple behaves as a conjugated protein in which retinene is the prosthetic group” [16]. As summarized by Hubbard in 1958, in the following years it became clear that rhodopsin is indeed “composed of a colorless protein, opsin, combined with the hindered 11-cis isomer of retinene”. The latter is isomerized by light to all-*trans* retinene, thus converting the dark adapted rhodopsin to lumirhodopsin and metarhodopsin, the form responsible for visual excitation [17]. We know that metarhodopsin is composed of the meta I and meta II states, with meta II being responsible for the transmission of the visual signal. The latter, at first, appeared to occur through coupling to an ATP-dependent cyclic nucleotide phosphodiesterase (PDE), as reported by Miki and coworkers in 1973 [18]. However, it was subsequently found that the coupling of rhodopsin to the PDE is dependent on GTP, rather than ATP. A substantial contamination of the ATP with GDP is what generated the artifact. Furthermore, as was hypothesized by Rodbell in 1971 for the glucagon-sensitive adenylyl cyclase [19], this PDE is not coupled directly to rhodopsin. It is a GTPase that actually links the activation of the receptor to the stimulation of the PDE that, in turn, catalyzes the hydrolysis of cyclic GMP to GMP [20,21]. This rhodopsin-coupled GTPase was later found by Fung and coworkers to be a heterotrimeric protein composed of α , β , and γ subunits, and dubbed by the authors transducin [22].

Throughout the 1950s and 1960s, evidence accumulated that rhodopsin is a structural component of the rod outer segment membrane, and that, actually, accounts for most of its proteic content [23]. It also became clear that retinal was covalently bound to opsin via a Schiff base [24-26]. The first molecular details of the picture, however, did not become evident until the 1980s, when Wand and Hargrave demonstrated that the Lys residue that serves as the attachment site is the 53rd amino acid from the C-terminus in the bovine receptor [27]. But the greatest advance brought by the 1980s was probably the disclosure of the complete amino acid sequence of bovine rhodopsin, which highlighted that the receptor consists of 7 transmembrane domains (7TMs). This milestone towards the understanding of the structure of GPCRs was achieved independently by the laboratories of Ovchinnikov, in the former Soviet Union, and Hargrave, in the United States [28,29]. In light of their previous finding that the C-terminus of the receptor is exposed to the cytoplasmic surface of the disk membrane, Hargrave and coworkers could sketch the first bi-dimensional model of rhodopsin, with the N-terminus in the extracellular milieu followed by a serpentine sequence that spans the membrane with 7 α -helices connected by three extracellular and three intracellular loops, leaving the C-terminus inside the cell [29]. A 7TM structure for bacteriorhodopsin had already been observed by means of electron microscopy [30]. However, bacteriorhodopsin is a light driven proton pump which bears only limited similarity to rhodopsin and the superfamily of GPCRs.

The coupling of sequence analysis and biochemical experiments can provide a significant amount of insights on the structural features of a protein. In light of the disclosure of the whole sequence of bovine rhodopsin, it became evident that the residue identified by Wand and Hargrave as the retinal attachment site corresponds to Lys 296 and is located in the seventh transmembrane domain (TM7), while, subsequent experiments by Sakmar and coworkers led to the discovery that Glu 113 in TM3 acts as the counterion for the protonated Schiff base [31]. But over ten years elapsed from the unraveling of rhodopsin's primary and secondary structure to the first direct experimental insights into its three-dimensional tertiary structure, which came in 1993, when Schertler and coworkers published a 9Å projection map of bovine rhodopsin obtained through electron crystallography of two-dimensional crystals [32]. Soon thereafter, Baldwin combined Schertler's findings with sequence analysis to allocate the individual TMs to the peaks of the projection map [33]. The resulting model, which provided the first three-dimensional arrangement of a GPCR helical bundle, has been extensively used

to infer structural information for other receptors as well, via sequence comparison and homology modeling [34]. This initial structural finding in 1993 was followed by a number of advancements achieved by the same group during the 1990s through electron cryo-microscopy, including a low resolution structure of bovine rhodopsin [35], two projection structures of frog rhodopsin [36], and the three-dimensional map of frog rhodopsin in 1997 [37]. With the latter, Unger and coworkers not only solved for the first time all seven helices, but also allowed the calculation of the approximate tilt angle of each helix.

The 1990s ended, and the new millennium started with another great milestone in GPCR structural studies: the issue of Science published on the 4th of August, 2000 contained the report, by Palczewski and coworkers, of the first high-resolution X-ray crystal structure of a GPCR (PDB ID: 1F88) [38]. The structure of dark-adapted bovine rhodopsin, representing the ground state of the receptor, had been solved with a 2.8 Å resolution, providing the first experimentally determined three-dimensional structure of a GPCR. Palczewski's structure unveiled the geometry of the 7TMs as well as the intracellular and extracellular loop domains almost in their entirety, revealing the conformation of the side-chains, and a number of intramolecular interactions. Besides confirming the helicity of the TMs and the packing geometry of the helical bundle predicted by Schertler's group, Palczewski's structure also revealed a β -hairpin conformation for the second extracellular loop, which hovers very low over the extracellular opening of the helical bundle, thus leaving retinal sheltered in a closed binding pocket. This feature, as subsequent publications will reveal, is typical of rhodopsin in all the forms crystallized up until now, but not of other GPCRs. Palczewski's crystal structure also confirmed the disulfide bridge that connects the extracellular side of TM3 with the second extracellular loop, a very common element of GPCR structure that had been first proposed at the end of the 1980s by Khorana and coworkers [39,40]. The role of this loop, especially in the C-terminal half, in recognition of small molecule ligands of the adenosine and P2Y nucleotide receptors has been probed [41]. Recently, evidence that this loop in rhodopsin might form a reversible gate that opens during the activation process was obtained through solid state ¹³C-NMR [42]. In particular, a coupling between movements of the second extracellular loop and TM5 and a rearrangement in the hydrogen-bonding networks connecting this loop with the extracellular ends of TM4, TM5 and TM6 have been detected upon the activation of rhodopsin. On the basis of data derived from computational simulations and mutagenesis experiments, we hypothesize that this role of the second extracellular loop might be common to other members of the superfamily as well [43-45].

Palczewski's original structure, as well as several refinements and different crystal forms subsequently published (PDB IDs: 1HZX, 1LH9, 1U19, 1GZM, 2I35, 2I36, 3C9L, 3PED) [46-51], shed light onto the topology of the dark-adapted rhodopsin, constrained in its inactive state by the bound 11-*cis*-retinal (9-*cis*-retinal in the case of 3PED). Despite that extensive breadth of the GPCR superfamily, these structures remained for several years the only example of crystallized GPCRs and were extensively employed as templates for the constructions of homology models, which provided the structural framework for the study of pharmaceutically relevant receptors and facilitated the discovery and the development of their ligands. These models were corroborated by a wealth of indirect experimental evidence, with mutation techniques amply applied to validate them based on predicted interactions of specific functionality of the ligand with amino acid residues of the receptor protein. For example, neoceptors, in which a small molecule ligand is chemically modified in a fashion complementary to the single amino acid mutation of a given GPCR, have been applied in this manner to the study of adenosine receptors [52]. However, although vastly supported by the experiments, up until recently the models could not be ultimately validated due to the lack of a second GPCR to be used as a reference, and a question remained as to the soundness of rhodopsin-based homology modeling. As we will discuss more amply in the next section, these doubts were dispelled by the recent disclosures of the structures of the β -adrenergic receptors,

and the A_{2A} adenosine receptor. In particular, a comparison between the crystal structure of the β_2 -adrenergic receptor in complex with carazolol (*vide infra*) with a rhodopsin-based model of the complex – exercise not possible before then – finally confirmed the applicability of homology modeling and molecular docking to the construction of GPCR-ligand complexes [53].

Electron paramagnetic resonance spectroscopy (EPR) experiments conducted by Farrens and coworkers had previously suggested that large molecular movements and rearrangements of the transmembrane domains accompanied the activation of the receptor [54]. In particular the authors suggested the requirement for the activation of rhodopsin of a distancing of TM3 from TM6, as corroborated by a number of biochemical experiments [54,55]. The full extent of the molecular changes that rhodopsin undergoes upon exposure to light remains yet to be elucidated at an atomic level, however substantial progress in this direction has been made in the last five years. Unlike the very unstable 3D crystals, 2D crystals of rhodopsin were found by Schertler, Vogel and coworkers to retain their crystalline order under illumination [56]. The analysis of these crystals through electron crystallography – which had already proven suitable for low resolution 3D determinations with ground state rhodopsin [57] – yielded the first 3D structure of the meta I photoactivated state of rhodopsin with a 5.5 Å resolution [58]. As mentioned, in the photoactivation process, the meta I state follows the bathorhodopsin and lumirhodopsin states and precedes the fully activated meta II state, which is responsible for the activation of transducin. The experiments of Schertler's group revealed that the formation of meta I rhodopsin does not involve large rigid body movements. The structural changes are instead circumscribed to the retinal binding pocket. Of note is that the electron density of Trp265 (6.48 according to the Ballesteros and Weinstein numbering), located in proximity of the kink of TM6, significantly deviates from the coordinates of the ground state structure, suggesting that the formation of meta I might be accompanied by a conformational rearrangement of this residue. Two years later, through solid-state NMR analysis of the meta II state of rhodopsin, Crocker and coworkers confirmed the conformational change of Trp265 concurrent with the activation of the receptor [59]. This observation is consistent with the toggle switch function attributed to aromatic residues located at this position not only for rhodopsin, but also for several other GPCRs, including adrenergic, adenosine, and P2Y receptors (*vide infra*) [60-62]. In line with Schertler's results for meta I, when Nakamichi and Okada solved, in 2006, the 3D crystal structures of bathorhodopsin (PDB ID: 2G87) and lumirhodopsin (PDB ID: 2HPY) [63,64], they did not find global conformational changes of the receptor with respect to the ground state. The two structures revealed that the bound retinal already abandons the 11-*cis* configuration in bathorhodopsin assuming a dihedral angle of about -155°, to undertake an almost complete transition to the all-*trans* configuration and a relaxation of the polyene chain in lumirhodopsin. The structure of lumirhodopsin also indicates a slight outward movement of the mid segment of TM3 in proximity of the retinal pocket. This subtle shift, however, does not yet produce any effect on the cytosolic side of the TM at this stage. Later the same year, Paczewski and coworkers made yet another advancement toward the determination of the structure of the activated rhodopsin with the publication of the 3D crystal structure of a deprotonated intermediate of the photoactivation process (PDB ID: 2I37) [50]. This intermediate, although showing spectral similarities with meta II, does not present the rigid body movements predicted by the above mentioned biophysical methods. The main differences with the ground state are to be found in the intracellular loops, which in the deprotonated intermediate appear significantly more disordered. As the authors clarify, the low resolution of the structure – 4.15 Å – PRN prevents a clear determination of the side chains. However, the predicted large rigid body movements, if present, would have been detected. A possible explanation that can be proposed is that the intermediate trapped by Paczewski, although close to the meta II state, has not yet undergone complete activation. This hypothesis would signify that the predicted vast rearrangement of the TMs occurs only upon completion

of the photoactivation process, hence leading to the conclusion that the activation of transducin is a rather sudden event rather than a gradual one.

Thus far, the closest crystallographic representation of the light-activated rhodopsin is to be found in the studies related to the unliganded opsin. In 2008, Ernst and coworkers crystallized native opsin in its empty state (PDB ID: 3CAP), and revealed an overall topological similarity with the retinal-bound rhodopsin, but with several substantial dissimilarities suggesting that the receptor might have been trapped in an activated state [65]. The noted differences included rearrangements of TM5 and TM6, especially on their intracellular sides, breakage of the ionic lock between TM3 and TM6, a feature that putatively constrains rhodopsin in its inactive conformation, and disruption of an aromatic interaction between TM7 and the adjacent cytoplasmic helix 8. Soon after, the authors published a further structure of opsin with a very similar conformation, but this time, in complex with a synthetic peptide corresponding to the extreme C-terminal fragment of the α subunit of transducin (PDB ID: 3DQB) [66]. The latter assumed an α -helical conformation with a C-terminal reverse turn and was found to be bound into the cavity formed by the outward tilt of TM6 and the pairing of TM5 and TM6. This information indeed confirmed the notion of a required substantial movement of the receptor's TM6 for it to activate its cognate G protein. According to the most recent double electron-electron resonance (DEER) measurements, the outward movement of this TM has been predicted to have an extent of about 5 Å and to be accompanied by smaller movements of TM1, TM7, and helix 8 [67].

As mentioned, the transduction of the visual signal in mammals is mediated by a specialized G protein named transducin. It is then reasonable to expect differences between the intracellular side of rhodopsin and the corresponding portion of receptors selective for other G protein subtypes. Moreover, the mechanism of activation envisioned for rhodopsin may also bear some peculiarities dependent on its unique coupling to transducin. However, invertebrate phototransduction, unlike its mammalian counterpart, occurs via G_q , making these systems appealing platforms for the study of a transduction pathway relevant for a variety of other receptors. On this front, a recent advance in the understanding of the structure-function relationships of GPCRs has been provided by the solution of the crystal structure of squid rhodopsin (PDB ID: 2Z73, 2Z1Y) [68,69], whose 2D crystals had been previously studied by Schertler and coworkers [70,71]. Unlike its mammalian homologue, this receptor shows a significant protrusion of the helical structures of TM5 and TM6 into the cytoplasm, which might be related to the structural requirements for coupling to G_q [68,69].

The reasons for which rhodopsin has emerged as a prototypical GPCR for structural studies and crystallographic analyses are multiple and not merely based on historical reasons. Above all they may be attributed to two factors: the structural rigidity conferred to the receptor by the covalently bound ligand and by the naturally high expression occurring in the rod cells of the retinas of various animals. As mentioned, recent advances have allowed the crystallization of the unliganded opsin without the presence of the rigidifying chromophore [65,66]. Always using rhodopsin as a benchmark, Schertler and coworkers also addressed the second problem, by solving the crystal structure of a recombinant rhodopsin mutant, bearing a stabilizing disulfide bridge between the N-terminus and the third extracellular loop and heterologously expressed in mammalian cells (PDB ID: 2J4Y, see also the alternative model 3C9M) [51,72]. In the last couple of years, the engineering of receptors with increased stability proved to be a viable route to solve the crystal structure of GPCRs. It has already led to the determination of the structures of the β -adrenergic receptors and the A_{2A} adenosine receptor (*vide infra*), and it is expected to yield further successes in the future.

Besides the crystallographic experiments, at the beginning of the millennium, Yeagle and coworkers attempted obtain an experimentally-based structure for the activated rhodopsin

through NMR spectroscopy [73,74]. Specifically, due to the difficulties inherent the analysis of membrane bound proteins, the authors devised a clever scheme based on the division of the sequence of the receptor into a series of overlapping peptides of which they gathered two dimensional homonuclear ^1H NMR spectra in solution. As a final step, the author computationally assembled the whole helical bundle on the basis of the available biophysical information, including not only the electron cryo-microscopy data gathered by Schertler and coworkers, but also electron paramagnetic spectroscopy and data from zinc crosslinking of histidine residues [37,54,55]. Using different sets of experimental data, the authors generated models for both the ground state and meta II rhodopsin (PDB IDs: 1JFP and 1LN6). These NMR-based models predicted a significant unwinding of the helices in the activated receptor, which however has not been confirmed by any of the crystallographic evidences, generating reasonable doubts on the robustness and reliability of this approach for the analysis of the membrane bound portions. Solution NMR experiments, however, may instead prove useful for the study of the soluble, generally nonconserved and often flexible, intracellular and extracellular domains (see Tikhonova and Costanzi, Unraveling the structure of G protein-coupled receptors through NMR, same issue of Current Pharmaceutical Design). These NMR-derived structures could then be combined with homology models of the helical bundles to generate hybrid experimental and computational structures, thus providing an effective way of dealing with the lack of sequence and structural conservation of these regions that prevents their modeling by homology. Alternatively, a more complete way to investigate the structure of GPCRs by NMR may be provided by solid-state techniques. Preliminary data let hope for future successes of these techniques in the analyses of whole receptors [75].

In addition to serving as a prototypical model for the study of the monomeric structure of the receptors, rhodopsin is also the only member of the superfamily whose oligomeric arrangement has been visualized experimentally. With a concerted effort of the research groups of Palczeski and Engel, the multimeric arrangement of rhodopsin and opsin in native mouse disc membranes has been directly observed by means of atomic-force microscopy (AFM) [76,77]. The low resolution image obtained indicated that rhodopsin molecules are closely packed together, forming dimers associated in rows of dimers that are then assembled into a paracrystalline layer of rows. Docking of the crystal structure of bovine rhodopsin into the AFM image led to a computational model (PDB ID: 1HZH) suggesting several intermolecular contacts that stabilize the multimeric arrangement [78]. In particular, the weakest contact is predicted to occur between TM1 and TM7 and to be responsible for the formation of the paracrystalline layer, while stronger contacts between the third intracellular loop and TM2 are proposed to be responsible for the formation of the rows of dimers. Finally, the strongest contacts, responsible for the dimeric interface, are predicted to occur between TM4 and TM5 of one monomer and the same TMs of another. This prediction is somewhat in contrast with an observation made by Schertler and coworkers on the basis of their cryo-microscopy map of squid rhodopsin [71], which had previously led the authors to the conclusion that the formation of rhodopsin dimers would involve only TM4-TM4 contacts, mediated by a side of the TM different than what later proposed by the AFM model. A subsequent experimentally supported model proposed by Guo and coworkers for the dopamine D2 receptors reconciled the two hypothesis, proposing that a rearrangement of the dimerization interface could be a critical component of activation, with the AFM and the cryo-microscopy models bearing clues of the inactive and active states, respectively [79]. Once again, the successive study of a variety of receptors, in light of discoveries previously made on rhodopsin, pointed towards a generalization of the conclusions, highlighting commonalities within the superfamily of GPCRs. Molecular modeling and biochemical experiments, in fact, suggested a similar dimerization interface for a variety of GPCRs, including, among others, the dopamine D2 receptor, and the $\text{A}_{2\text{A}}$ adenosine receptor [79-82]. Moreover, recent evidence from a combination of bioluminescence and fluorescence protein complementation combined with energy transfer approaches led to the

demonstration of a higher order oligomeric arrangement also for the dopamine D2 receptor, involving at least the association of four models [83].

The crystal structure of the β -adrenergic receptors and A_{2A} adenosine receptor: Rhodopsin is not alone

With the advent of molecular cloning and genome sequencing, it became evident that rhodopsin is not a structurally and functionally unique receptor, but rather it is a member of a large superfamily that in the human genome counts over 800 receptors [84]. Testifying to the ancient evolutionary origins of GPCR signaling, genes encoding for these receptors have been identified not only in mammals and vertebrates, but also in a variety of additional genomes, including, among others, those of plants, worms and fungi [85].

By the end of the 1970s, clear analogies had emerged between the system composed by light, rhodopsin, G protein, and the GTP dependent phosphodiesterase on one hand and, on the other hand, those more in general composed by hormones, hormone receptors, G proteins, and adenylate cyclase [86,87]. The first evidence of a structural platform common to the GPCR superfamily, however, arose only in the mid to late 1980s, when the laboratories of Lefkowitz and Strader published the cloning of the hamster β_2 -adrenergic receptor. These data indicated a significant sequence similarity with bovine rhodopsin, and unveiled that also the newly cloned receptor was endowed with a seven membrane spanning topology [88,89]. Twenty years elapsed from this discovery to the crystallization of the human β_2 -adrenergic receptor, which ultimately sealed the long postulated structural homology. The first structures to be disclosed, in the fall of 2007, were obtained by the groups of Kobilka and Schertler, crystallizing the receptor in a complex with carazolol – a partial inverse agonist – and the fragment antigen binding (Fab) of a monoclonal antibody that recognizes the third intracellular loop (2R4S, 2R4R) [90,91]. Soon after, a higher resolution structure has been published by the groups of Kobilka and Stevens (2RH1) [92,93]. This new structure, which unlike the first one that had a defined electron density for the extracellular portion of the receptor and for the ligand, was obtained through the generation of a fusion protein in which most of the third intracellular loop of the receptor had been replaced with the T4-lysozyme, a well crystallizable protein. Also in this case, these breakthroughs revealed a very good similarity in the topology of the helical bundles of the β_2 -adrenergic receptor and rhodopsin, with an RMSD of 2.7 Å between the C α atoms of all residues in the transmembrane regions. The papers examined in great detail similarities and differences between the two systems, including a thorough comparison of the ligand binding pockets [92]. In this regard, the new data revealed a striking overlap between the crystallized retinal and carazolol, but also identified one main difference: the second extracellular loop, which in rhodopsin forms a β -strand and hovers very low over the binding pocket, closing its access from the extracellular side, in the β_2 -adrenergic receptor has a completely different topology that renders the pocket open and readily accessible by the ligands and includes a short α -helical segment. Following the advances of 2007, two additional structures were published in 2008. The first was another structure of the human β_2 -adrenergic receptor fused with the T4-lysozyme, this time bearing a stabilizing point mutation and solved in complex with the inverse agonist timolol, published by the group of Stevens [94]. Notably, this structure suggested the presence of a cholesterol molecule in a specific binding pocket located between TM2, TM3, and TM4. The second structure was published by the group of Schertler, and referred to the turkey β_1 receptor stabilized by a number of mutations and solved in complex with the antagonist cyanopindolol [95]. In line with the significant sequence identity (64 % [96]), the structure of the turkey β_1 receptor was remarkably similar to that of the human β_2 receptor, the only major difference being the presence of an α -helical structure in the second extracellular loop in the former, but not in the latter structure. In the fall of 2008, a fourth GPCR structure, namely the A_{2A} adenosine receptor in complex with the potent antagonist 4-2-[7-amino-2-(2-furyl)-1,2,4-triazolo[1,5-a][1,3,5]triazin-5-yl-amino]ethylphenol (ZM241385),

was solved through X-ray crystallography by the groups of Stevens and IJzerman and confirmed once again the structural homology of the GPCR superfamily, in particular with respect to the helical bundle [97]. The structure also confirmed the idea that the topology of the second extracellular loop is probably unique to each particular receptor. In the case of the A_{2A} receptor, this domain assumes a conformation that is distinct from the one displayed by rhodopsin or the β_2 -adrenergic receptor. However, as in the β_2 receptor, the second extracellular loop of the A_{2A} receptor does not block the access to the entrance of the ligand binding pocket from the exofacial side of the helical bundle, as it does in rhodopsin. Curiously, the putative ionic lock between TM3 and TM6 seen in the rhodopsin structure and hypothesized to indicate the inactive conformation of a receptor did not appear in the structures of either the β adrenergic receptors or the A_{2A} adenosine receptor. Additionally, the crystal structure of the A_{2A} receptor revealed a ligand binding mode somewhat different from that seen in rhodopsin and the β -ARs, with ZM241385 binding in an extended conformation and perpendicular to the plane of the membrane. Thus, besides confirming the many structural commonalities of GPCRs, the crystal structure of the A_{2A} receptor also highlighted that each receptor has its own peculiar characteristics, with obvious consequences on molecular modeling and drug discovery.

Conclusions

Through this paper, we have reviewed, in a historical perspective, the role of rhodopsin as a structural prototype for the study of the entire superfamily of GPCRs. Insightful reviews on rhodopsin studies have been published by Palczewski and coworkers [98-100]. With such a large superfamily of receptors and so little structural information, homology modeling and molecular docking have been applied for years to the study of GPCR-ligand complexes, all based on the various structures of rhodopsin as the sole template. As reviewed in an highly informative article by Hanson and Stevens, breakthroughs in the last couple of years have elucidated the structures of additional receptors, namely the β_1 - and β_2 -adrenergic receptors and the A_{2A} adenosine receptor [96]. Given the impressive amount of biochemical and biophysical data that has been obtained for them, it was perfectly natural, as explained by Lefkowitz and coworkers, that the β -adrenergic receptors became in 1986 the first receptors whose structural similarity to rhodopsin was appreciated based on sequence analysis and, twenty one years later, also became the first to join rhodopsin in the Olympic company of crystallized GPCRs [101]. The availability of these structures in addition to those for rhodopsin, now offer the unique opportunity of gauging the accuracy of molecular modeling techniques and perfecting the computational protocol. Toward this purpose, a comparison of the crystal structure of complex with carazolol in the β_2 -adrenergic receptor with an *in silico* model based on homology to rhodopsin offered the first direct evidence of the applicability of homology modeling and molecular docking to the generation of accurate three-dimensional structures and the study of receptor-ligand interactions [53]. Moreover, subsequent to the crystallization of the A_{2A} adenosine receptor, Stevens and coworkers organized the first “critical assessment of GPCR structural modeling and docking”, the results of which highlighted that the construction of accurate models, although challenging, is certainly achievable [102]. The docking of the ligands and the scoring of the poses clearly emerged as the most difficult components. However, it is very encouraging that accurate models capturing most of the receptor-ligand interactions have been submitted by a handful of groups participating in this *in silico* binding experiment. Another insightful analysis of homology models of the adenosine receptors in light of the new A_{2A} crystal structure has been published by Ivanov and coworkers, who also confirmed the usefulness of GPCR modeling, especially when supported by experimental data, and suggested its applicability also to the study of the binding mode agonists [103].

Considering the new pace of the field and the number of receptors currently under investigation, it is reasonable to expect that crystal structures for additional members of the superfamily will

be solved soon. As discussed, the structures so far elucidated are mostly related to the inactive conformations of the receptors. Certainly, a further goal in the field is to derive the structure of receptors in their signaling state, possibly in complex with agonists. Rhodopsin is no longer the lone template for modeling, but will certainly keep serving the GPCR community as a prototypical receptor and a generator of ideas to be tested on other systems. These advances, coupled with the introduction of more sophisticated modeling algorithms and the increase in computer power, raise the expectation for a substantial boost of the robustness and accuracy of computer-aided drug discovery techniques in years to come.

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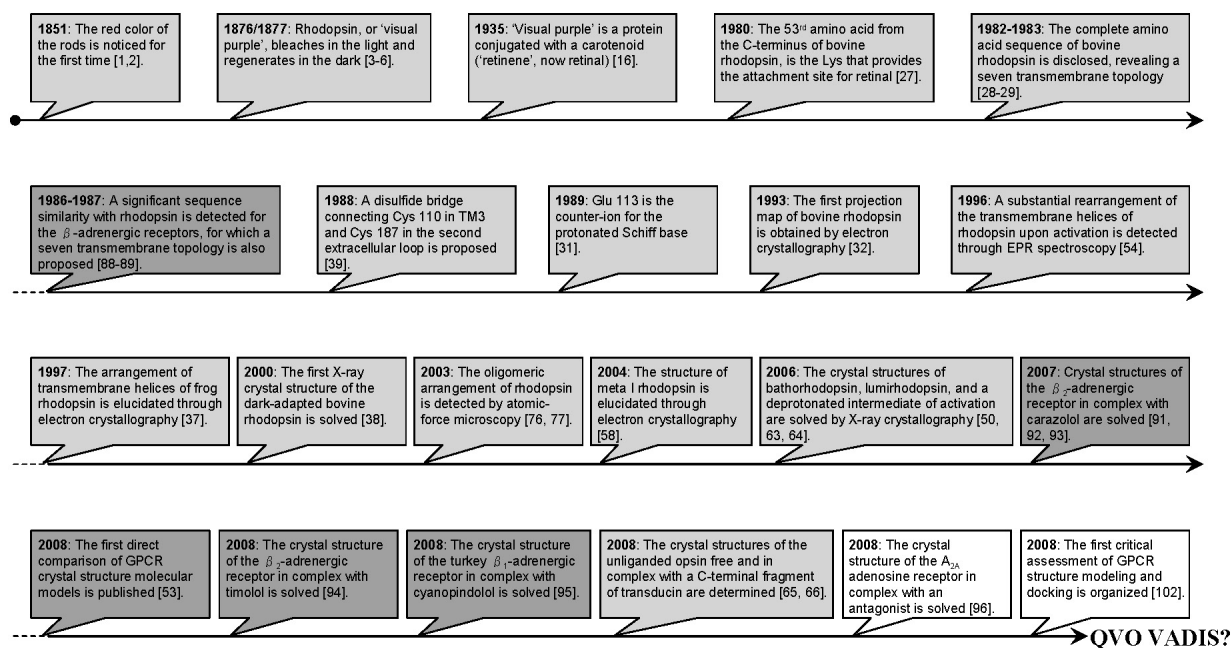


Figure 1.

A schematic timeline representing some of the most significant milestones in the structural studies of GPCRs – light gray boxes refer to rhodopsin; dark gray boxes refer to the β -adrenergic receptors; white boxes refer to the adenosine A_{2A} receptor.